



| | |
|----------------|---------------|
| Receipt number | 662-06-E-4225 |
| Study number | 94225 |

FINAL REPORT

A 96-hour Acute Toxicity Study of 13F-SFA with Medaka

July 25, 2007

Kurume Laboratory
Chemicals Evaluation and Research Institute, Japan

STATEMENT

Kurume Laboratory
Chemicals Evaluation and
Research Institute, Japan

Sponsor DAIKIN INDUSTRIES, LTD.

Title A 96-hour Acute Toxicity Study of 13F-SFA with Medaka

Study number 94225

I, the undersigned, hereby declare that this report provides a correct English translation of the Final Report (Study No. 94225, issued on July 25, 2007)

Date

October 1, 2009

Study Director

Makiko Anai

Makiko Anai

GLP STATEMENT

Kurume Laboratory
Chemicals Evaluation and
Research Institute, Japan

Sponsor DAIKIN INDUSTRIES, LTD.

Title A 96-hour Acute Toxicity Study of 13F-SFA with Medaka

Study number 94225

The study described in this report was conducted in compliance with the following GLP principles:

- (1) "Standard Concerning Testing Facility Relating to New Chemical Substances" (November 21, 2003; No. 1121003, Pharmaceutical and Food Safety Bureau, Ministry of Health, Labour and Welfare; November 17, 2003, No. 3, Manufacturing Industries Bureau, Ministry of Economy, Trade and Industry; No. 031121004, Environmental Policy Bureau, Ministry of the Environment)
- (2) "OECD Principles of Good Laboratory Practice (November 26, 1997, ENV/MC/CHEM (98)17)"

This final report reflects the raw data accurately and it has been confirmed that the test data are valid.

Date July 25, 2007

Study Director _____
 Signed in original
 Makiko Anai

QUALITY ASSURANCE STATEMENT

Kurume Laboratory
Chemicals Evaluation and
Research Institute, Japan

Sponsor DAIKIN INDUSTRIES, LTD.

Title A 96-hour Acute Toxicity Study of 13F-SFA with Medaka

Study number 94225

I assure that the final report accurately describes the test methods and procedures, and that the reported results accurately reflect the raw data of the study.

The inspections and audit of this study were carried out and the results were reported to the Study Director and the Test Facility Management by Quality Assurance Unit as follows.

| Item of inspection / audit | Date of inspection / audit | Date of report to Study Director and Test Facility Management |
|--|----------------------------|---|
| Study plan draft | June 1, 2007 | June 1, 2007 |
| Study plan | June 1, 2007 | June 1, 2007 |
| Amendment of study plan | July 25, 2007 | July 25, 2007 |
| Measurement of solubility | June 4, 2007 | June 5, 2007 |
| | June 5, 2007 | June 5, 2007 |
| Start of the exposure and after the exposure | June 4, 2007 | June 8, 2007 |
| | June 8, 2007 | June 8, 2007 |
| Raw data and final report draft | July 24, 2007 | July 24, 2007 |
| Final report | July 25, 2007 | July 25, 2007 |

Date

July 25, 2007

Head of Quality Assurance Unit

Signed in original

Keiji Shiraishi

CONTENTS

| | Page |
|--|--|
| Title | 1 |
| Sponsor | 1 |
| Test facility | 1 |
| Objective | 1 |
| Test method | 1 |
| Applied GLP | 1 |
| Dates | 2 |
| Storage of test item, raw data, etc. | 2 |
| Personnel | 3 |
| Approval of final report | 3 |
| SUMMARY | 4 |
| 1. Test item | 6 |
| 2. Test sample | 7 |
| 3. Test materials and methods | 8 |
| 4. Results and discussion | 12 |
| 5. Factors that affected reliability of test results | 14 |
| 6. Content of deviation from protocol | 14 |
| | |
| Tables | |
| Table 1 | Cumulative mortality |
| Table 2 | Observed abnormal response |
| Table 3-1 | Dissolved oxygen concentration of test solutions |
| Table 3-2 | pH of test solutions |
| Table 3-3 | Temperature of test solutions |
| Table 4 | LC ₅₀ to Medaka |
| Appendix 1 | Chemical characteristics of dilution water |
| Appendix 2 | Analytical method and measured concentration of test item and 13F-EtOH (hydrolyzed product) |
| Appendix 3 | Calibration curve and chromatogram |
| Appendix 4 | Solubility in dilution water |
| Additional data | Results of preliminary studies |

| | |
|---------------|--|
| Title | A 96-hour Acute Toxicity Study of 13F-SFA with Medaka |
| Sponsor | DAIKIN INDUSTRIES, LTD. 1-1, Nishi-Hitotsuya, Settsu, Osaka 566-8585, Japan |
| Test facility | Kurume Laboratory Chemicals Evaluation and Research Institute, Japan 3-2-7 Miyanojin, Kurume-shi, Fukuoka 839-0801, Japan |
| Objective | The purpose of this study is to determine the acute toxicity of 13F-SFA on fish. |
| Test method | This study was performed according to the following test methods and guidance document. <ol style="list-style-type: none">(1) Fish, Acute Toxicity Test stipulated in the "Testing Methods for New Chemical Substances" (November 21, 2003; No. 1121002, Pharmaceutical and Food Safety Bureau, Ministry of Health, Labour and Welfare; November 13, 2003, No. 2, Manufacturing Industries Bureau, Ministry of Economy, Trade and Industry; No. 031121002, Environmental Policy Bureau, Ministry of the Environment)(2) OECD Guidelines for Testing of Chemicals, Section 2 : Effects on Biotic Systems, 203 Fish, Acute Toxicity Test (Guideline 203, 1992)(3) OECD Guidance Document No.23 "Guidance Document on Aquatic Toxicity Testing of Difficult Substance and Mixtures" (September 2000) |
| Applied GLP | This study was conducted in compliance with the following GLP principles: <ol style="list-style-type: none">(1) "Standard Concerning Testing Facility Relating to New Chemical Substances" (November 21, 2003; No. 1121003, Pharmaceutical and Food Safety Bureau, Ministry of Health, Labour and Welfare; November 17, 2003, No. 3, Manufacturing Industries Bureau, Ministry of Economy, Trade and Industry; No. 031121004, Environmental Policy Bureau, Ministry of the Environment)(2) "OECD Principles of Good Laboratory Practice (November 26, 1997, ENV/MC/CHEM(98)17)" |

Dates

| | |
|----------------------------------|---------------|
| Study initiation date | June 1, 2007 |
| Experimental starting date | June 4, 2007 |
| Solubility study starting date | June 4, 2007 |
| Bioassay starting date | June 4, 2007 |
| Experimental completion date | June 8, 2007 |
| Solubility study completion date | June 5, 2007 |
| Bioassay completion date | June 8, 2007 |
| Study completion date | July 25, 2007 |

Storage of test item, raw data, etc.

(1) Test item

The test sample^{*1} will be sealed in a storage vessel and stored in archives in this laboratory for ten years after the receipt of notice specified under Clause 1 or Clause 2 in Article 4, Clause 2 or Clause 3 or Clause 8 in Article 4-2, and Clause 2 in Article 5-4 or Clause 2 in Article 24 or Clause 2 in Article 25-3 of "Law Concerning Examination and Regulation of Manufacture, etc. of Chemical Substances". If it is not stable for the storage period, it is stored as long while it is kept stable. Treatment of the sample after the storage period will be discussed with sponsor.

*1 It will be stored as the common sample for storage of these studies (Study Nos. 94224, 94225 and 94226).

(2) Raw data and materials

Raw data, the study plan, documents concerning the study presented by the sponsor, the final report and necessary materials will be stored in archives in this laboratory for ten years after the receipt of the notice specified under Clause 1 or Clause 2 in Article 4, Clause 2 or Clause 3 or Clause 8 in Article 4-2, and Clause 2 in Article 5-4 or Clause 2 in Article 24 or Clause 2 in Article 25-3 of "Law Concerning Examination and Regulation of Manufacture, etc. of Chemical Substances". Treatment of raw data and materials, etc. after the storage period will be discussed with sponsor.

Personnel

Study Director : Makiko Anai
Section 4 (Eco-toxicity test area)

Study personal

Biology : Miyuki Narasaki, Takeshi Matsuura,
Makiko Anai, Junji Azuma,
Yumika Hiroo

Analytical chemistry : Ryuta Adachi, Tomoyo Kagamiyama,
Hitomi Inoue, Hiroko Sugimoto,
Mika Ono

Approval of final report

Study Director

Date

July 25, 2007

Signature

Signed in original

Makiko Anai

SUMMARY

Title

A 96-hour Acute Toxicity Study of 13F-SFA with Medaka

Test conditions

| | |
|--|---|
| (1) Test item | 13F-SFA |
| (2) Test organism | Medaka (<i>Oryzias latipes</i>) |
| (3) Exposure duration | 96 hours |
| (4) Test concentration | The solution which collected from the middle layer in the suspension of the test item (nominal concentration: 100 mg/L) and control |
| (5) Replicate | Two replicates/test level |
| (6) Number of organism | Ten fish / test level (five fish / test vessel) |
| (7) Dilution water | Dechlorinated tap water |
| (8) Type of test | Closed Semi-static (renewal at every 24 hours) |
| (9) Preparation of test solution | The test sample and dilution water were mixed to produced about 100 mg/L (nominal concentration) and they were stirred under closed system for about 24 hours. After settlement for 1 hour, test solution was prepared by taking out from the middle layer. |
| (10) Volume of test solution | About 6 L / test level (About 3 L / test vessel) |
| (11) Temperature of test solutions | 24±1°C |
| (12) Irradiation condition | Artificial light of white fluorescent lamp, 16-hour light / 8-hour dark |
| (13) Feeding | No feeding |
| (14) Aeration | No aeration |
| (15) Analysis of concentration of test item and 13F-EtOH (hydrolyzed product) in test solution | GC analysis (at the start of the exposure, before and after the renewal, and the end of the exposure) |

Results

| | |
|--|---|
| (1) Solubility of test item in dilution water (24±1°C) | 0.126 mg/L |
| (2) Concentration of test item in test solution (Percentage of concentration at preparation) | |
| At the start of the exposure and after the renewal | 0.391 to 1.15 mg/L |
| Before the renewal and at the end of the exposure | 0.0689 to 0.204 mg/L (17.6 to 46.5%) |
| (3) Concentration of 13F-EtOH in test solution | |
| At the start of the exposure and after the renewal | 0.0179 to 0.0442 mg/L |
| Before the renewal and at the end of the exposure | 0.0811 to 0.248 mg/L |
| (4) 96-hour LC ₅₀ (Median Lethal Concentration) | > 0.306 mg/L |

[The values of (4) is based on geometric mean of the measured concentrations.]

Conclusion

This study was conducted as a limit test at the concentration around solubility of the test item in dilution water to confirm the effect on the test organisms. It was concluded that the test item has no acute toxicity to the test organisms at the concentration around water solubility, since the measured concentrations of the test solutions at the preparation were around the solubility in dilution water and no effect on the test organisms was observed under the test condition.

1. Test item

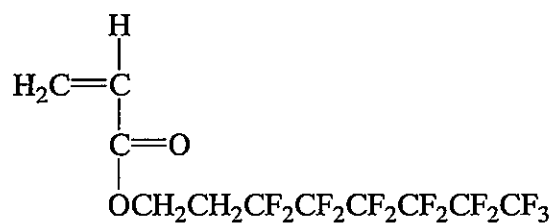
In this report, 13F-SFA has the following name etc.

1.1 Chemical name*²

3,3,4,4,5,5,6,6,7,7,8,8,8-tridecafluorooctyl acrylate

1.2 Chemical structure etc.*²

Structural formula



Molecular formula $\text{C}_{11}\text{H}_7\text{F}_{13}\text{O}_2$

Molecular weight 418.15

CAS Number 17527-29-6

*² Information supplied by the sponsor

2. Test sample

2.1 Supplier and lot number^{*2}

| | |
|------------|-------------------------|
| Supplier | DAIKIN INDUSTRIES, LTD. |
| Lot number | 6X002 |

2.2 Purity^{*2}

| | |
|-----------|------------------------------------|
| Test item | 99.7% |
| Impurity | Unknown constituent component 0.3% |

2.3 Confirmation of test item

It was confirmed that infrared (IR) spectrum of the test item provided by the sponsor coincided with IR spectrum analyzed in this laboratory.

2.4 Physicochemical properties^{*2}

| | | |
|----------------------------------|--------------------------------|--------------------------|
| Appearance at normal temperature | Colorless and clear liquid | |
| Boiling point | 78°C (8 mmHg) | |
| Density | 1.554 g/cm ³ (25°C) | |
| Solubility | Water | Insoluble |
| | Dimethylsulfoxide | Soluble (fully miscible) |
| | Acetone | Soluble (fully miscible) |

*2 Information supplied by the sponsor

2.5 Storage condition and confirmation of stability at storage condition

| | |
|---------------------------|--|
| Storage condition | Dark storage place at room temperature |
| Confirmation of stability | The stability of the test item during the test period was confirmed by no alteration in the IR spectra of the test item before the experimental start and after the experimental completion. |

3. Test materials and methods

3.1 Test organism

(1) Species

Medaka (*Oryzias latipes*)

(2) Reason for selection of species

This species is recommended in the test guidelines.

(3) Size

Total length 2.3±1.2 cm

Size of test organism was applied the regulated size set to test method (1).

(4) Supplier

Kurume Laboratory, Chemicals Evaluation and Research Institute, Japan
3-2-7 Miyanojin, Kurume-shi, Fukuoka 839-0801, Japan

(5) Acclimation

Medaka hatched out on December 11, 2006 were acclimated for 14 days by flow-through condition under the same water quality (dechlorinated tap water), temperature (24±1°C), light and dark period (16-hour light / 8-hour dark) as test condition (on May 21 to June 4, 2007). The mortality was 0% during the 7 days before the start of the exposure. The test organism at the start of the exposure was 5-month-old fish. The test organisms were not treated with a medicament for external disinfection. The test organisms were fed the feed mixture for carp (2C), and not fed for 24 hours before the start of the exposure. Dissolved oxygen concentration in breeding water during acclimation was kept not less than 80% of air saturation value. A 96-hour acute toxicity test of CuSO₄·5H₂O (Reagent chemical, Wako Pure Chemical Industries, Ltd.) to confirm reproducibility of the test system was carried out on May 21 to May 25, 2007 and the 96-hour LC₅₀ was 0.445 mg/L. This value was within the stipulated range (mean ± 2S.D. : 0.124 to 0.978 mg/L) [mean ± S.D. : 0.551 ± 0.214 mg/L (n=38)] to background data in this laboratory. All of the values shown above for the reference substance were converted into CuSO₄ value.

(6) Allocation to the test groups

Medaka were allocated at random to each test group.

3.2 Dilution water

Dechlorinated tap water, aerated sufficiently and controlled temperature, was used. Some chemical characteristics of the dilution water measured regularly are listed in Appendix 1.

3.3 Test apparatus and equipment

(1) Test apparatus

Test vessel : 3 L Glass tank (diameter: 16 cm, depth: 17 cm)

The test vessels were covered with lid in order to prevent dust, and volatilization of the test solution and closed without headspace.

(2) Test equipment

Water bath : Plastic tank

Warming / cooling unit (Type HCA 250, Sato craft)

3.4 Test conditions

(1) Conditions of exposure

(a) Type of test

The test organisms were exposed to the test solution containing the test item.

The test solutions were renewed at every 24 hours, as closed semi-static regime.

(b) Exposure duration

96 hours

(c) Test concentration

Based on the results of the preliminary studies, it was expected that the test solution at around the solubility in dilution water would have no effect on the test organisms. Therefore, the definitive study was conducted as the limit test with suspended solution which was prepared by taking out from the middle layer of 24-hour mixed solution (nominal concentration: 100 mg/L). The results of the preliminary studies are shown in Additional data.

(d) Control

The dilution water without the test item, which was treated in the same manner as the test solution, was used as the control.

- (e) Replicates
Two replicates / test level
- (f) Number of organism
Ten fish / test level (five fish / test vessel)
- (g) Volume of test solution
About 6 L / test level (about 3 L / test vessel)

(2) Conditions of test environment

- (a) Water temperature
24±1°C
- (b) Dissolved oxygen concentration
The test was performed in the condition where dissolved oxygen concentration was at least 60% or more of the saturated concentration at the water temperature. Aeration was not used during the exposure.
- (c) pH
The test was performed without adjusting pH.
- (d) Irradiation condition
Artificial light of white fluorescent lamp, 16-hour light/8-hour dark
- (e) Feeding
Test organisms were not fed during the exposure.

3.5 Preparation of test solution

No correction with purity was done for the preparation of the test concentration. The test sample was employed in terms of volume using the density [1.554 g/cm³ (25°C)] for the preparation of test solution.

After the test sample was added into the dilution water filled in Erlenmeyer flask with micro volumeter (Eppendorf Co., Ltd) to prepare test solution of 100 mg/L as nominal concentration, the flask was immediately sealed with a plug not to produce head space. The solution was gently stirred by magnetic stirrer for about 24 hours to produce dispersed solution with suspended test item. After cease of stirring, the solution was settled for 1 hour and then test solution was prepared by taking out from the middle layer of the settled solution. The prepared test solution was immediately divided into each test vessel and covered with glass lid not to produce head space.

3.6 Observation and measurements

(1) Observation of test organisms

Mortality and visible abnormality were observed at 3, 24, 48, 72 and 96 hours after the start of the exposure. A fish was considered as dead if the observable motion (motion of mouth and opercula etc.) were not observed and touching of the caudal peduncle with glass rod produced no reaction.

(2) Total length and body weight of test organism

The test organisms in the control group were used for measuring total length and body weight after the end of the exposure.

(3) Appearance of test solution

Appearance of the test solutions were observed at the start of the exposure and before the renewal (after 24 hours).

(4) Condition of test solutions

Dissolved oxygen concentration, pH and temperature of the test solutions were measured at the start of the exposure, before and after the renewal and the end of the exposure. At the preparation, another solution sampled from the container for preparation was used for the measurement. At 24 hours after, the measurement was carried out for one test vessel in each level. The dissolved oxygen concentration measurements were carried out with an oxygen meter (YSI Incorporated., YSI Model 58). The pH measurements were carried out with a portable pH meter (DKK-TOA, Model HM-21P). The temperature measurements were carried out with a calibrated red alcohol thermometer of glass stick type.

(5) Concentration of test item in test solution

The concentration of the test item in the test solutions was measured at the start of the exposure, before and after the renewal and the end of the exposure. Since the test item was hydrolyzed and produced 2-(perfluorohexyl) ethanol (abbreviation: 13F-EtOH which is the test item of study number 94232 to 94234), the concentration of 13F-EtOH was also measured. At the start of the exposure and after the renewal (at the preparation) another solution sampled from the container for preparation was used for analysis. Before the renewal and the end of the exposure, the test solution for analysis was taken out with equal volume from the middle layer of the test solution in test vessels in each test level, and mixed. The concentrations of the test item and 13F-EtOH were analyzed by gas chromatography (GC). Analytical method and measured concentration of test item and 13F-EtOH are shown in Appendix 2, and analytical calibration curve and chromatograms are shown in Appendix 3.

(6) Solubility of test item in dilution water

Since the solubility of the test item was expected less than 100 mg/L, it was measured concurrently with the definitive study. The detail and the result of the measurement of the solubility are shown in Appendix 4.

3.7 Calculating method of LC₅₀^{*3}

The LC₅₀ values were estimated as “> the test concentration” since no less than 50% of mortality was observed in the exposure level.

The results of the study were estimated based on a geometric mean of the measured concentrations as the test concentration.

*3 LC₅₀ (Median Lethal Concentration): The test item concentration at which 50% of the test organisms causes mortality during the exposure.

3.8 Validity of the test

(1) The mortality in the control should not exceed 10%.

(2) Dissolved oxygen concentration must be at least 60% of the air saturation value at the water temperature in the test during exposure.

3.9 Treatment of numerical values

Values were rounded off in accordance with JIS Z 8401 rule B, 1999.
(JIS; Japanese Industrial Standards)

4. Results and discussion

4.1 Mortality

The mortality during the exposure was not observed in the exposure level. Cumulative mortality of each exposure period was shown in Table 1. The mortality in the control was not observed, which meets the criterion for the validity of the test (i.e. not more than 10%).

4.2 Observed abnormal response

No abnormal responses were observed in the control.

The results of observation were based on the comparison with the control organisms. No abnormal responses were observed in the exposure level during exposure. The abnormal responses observed during the exposure are shown in Table 2.

4.3 Size of test organism [Mean ± Standard deviation (n=10)]

| | |
|--------------|--------------|
| Total length | 2.6±0.12 cm |
| Body weight | 0.16±0.018 g |

4.4 Observation and measurement of test solution

(1) Appearance of test solution

The test solutions were clear and colorless at the start of the exposure and before the renewal.

(2) Condition of test solutions

The measured values of dissolved oxygen concentration, pH and temperature during the exposure ranged from 6.4 to 8.5 mg/L, 7.3 to 7.8 and 24.1 to 24.7°C, respectively. Conditions of the test solutions are shown in Tables 3-1, 3-2 and 3-3. The measured values of dissolved oxygen concentration met the criterion for the study validity (at least 60% or more of saturate concentration*⁴ at the water temperature).

*4 Saturated dissolved oxygen concentration (23 to 25°C): 8.39 to 8.11 mg/L
(JIS K 0102, 1998)

(3) Concentration of test item in test solution

The measured concentrations of the test item in the test solution were 0.391 to 1.15 mg/L at the start of the exposure and after the renewal. Before the renewal and at the end of the exposure, they were 0.0689 to 0.204 mg/L which were 17.6 to 46.5% of the concentration at the preparation. The measured concentrations of 13F-EtOH in the test solution were 0.0179 to 0.0442 mg/L at the start of the exposure and after the renewal, 0.0811 to 0.284 mg/L at before the renewal and at the end of the exposure. The results of the measured concentrations of the test item and 13F-EtOH are shown in Appendix 2.

4.5 LC₅₀

Both of the 48 and 96-hour LC₅₀s of the test item to Medaka were >0.306 mg/L. The LC₅₀s at each time are shown in Table 4.

4.6 Discussion

This study was conducted as a limit test in order to confirm the effect of the test item on the test organisms at the concentration around the solubility of the test item in dilution water. Although all measured concentrations of the test item in the test solution at the preparation during the exposure were greater than the solubility measured concurrently with the definitive study, they decreased at the 24 hours after the preparation. The reason why the concentration of the test item in the test solution decreased might be due to the characteristics of volatile of the test item and hydrolysis of the test item proceeded gradually with time during the exposure. This study was performed using semi-static replace regime at every 24 hours in order to hold the concentration of the test item as much as possible. Therefore, this study is thought to be appropriate as a test with the concentration around the solubility in the dilution water. This definitive study complied with the test conditions since the environmental conditions in the test were also within the suitable range. Since no adverse effect was found under the condition in the definitive study, it was concluded that the test item had no adverse acute effect on the test organisms at the concentration around the solubility in dilution water. In addition, it is supposed 13F-EtOH of the hydrolyzed product had the additive effect on the test organisms since no adverse effect was observed in the study although the measured concentrations of that in the test solution were 0.0179 to 0.284 mg/L.

5. Factors that affected reliability of test results

There were no factors which might have affected the reliability of the test.

6. Content of deviation from protocol

None

Table 1 Cumulative mortality

| Measured concentration *5 (mg/L) | Cumulative mortality (%) | | | | |
|-------------------------------------|--------------------------|----------|----------|----------|----------|
| | 3 hours | 24 hours | 48 hours | 72 hours | 96 hours |
| Control | 0 | 0 | 0 | 0 | 0 |
| 0.306 | 0 | 0 | 0 | 0 | 0 |

*5 Geometric mean of the measured concentrations
(The followings are expressed as measured concentration)

Table 2 Observed abnormal response

| Measured concentration (mg/L) | Result of observation (Left column: Number of affected fish/Total survival number, Right column: Symptom detail) | | | | | | | | | |
|----------------------------------|---|------|----------|------|----------|------|----------|------|----------|------|
| | 3 hours | | 24 hours | | 48 hours | | 72 hours | | 96 hours | |
| | Control | 0/10 | N | 0/10 | N | 0/10 | N | 0/10 | N | 0/10 |
| 0.306 | 0/10 | N | 0/10 | N | 0/10 | N | 0/10 | N | 0/10 | N |

N : Normal (No abnormal response)

Table 3-1 Dissolved oxygen concentration of test solutions

| Measured concentration (mg/L) | 0 hour | 24 hours | | 48 hours | | 72 hours | | 96 hours |
|-------------------------------|--------------|--------------------|-------------------|--------------------|-------------------|--------------------|-------------------|------------|
| | At the start | Before the renewal | After the renewal | Before the renewal | After the renewal | Before the renewal | After the renewal | At the end |
| Control | 8.4 | 6.7 | 8.2 | 6.8 | 8.2 | 7.0 | 8.4 | 6.9 |
| 0.306 | 8.4 | 6.7 | 8.0 | 6.4 | 8.2 | 6.6 | 8.5 | 6.7 |

Unit : mg/L

Table 3-2 pH of test solutions

| Measured concentration (mg/L) | 0 hour | 24 hours | | 48 hours | | 72 hours | | 96 hours |
|-------------------------------|--------------|--------------------|-------------------|--------------------|-------------------|--------------------|-------------------|------------|
| | At the start | Before the renewal | After the renewal | Before the renewal | After the renewal | Before the renewal | After the renewal | At the end |
| Control | 7.6 | 7.4 | 7.7 | 7.5 | 7.7 | 7.4 | 7.7 | 7.4 |
| 0.306 | 7.7 | 7.3 | 7.8 | 7.4 | 7.8 | 7.3 | 7.8 | 7.3 |

Table 3-3 Temperature of test solutions

| Measured concentration (mg/L) | 0 hour | 24 hours | | 48 hours | | 72 hours | | 96 hours |
|-------------------------------|--------------|--------------------|-------------------|--------------------|-------------------|--------------------|-------------------|------------|
| | At the start | Before the renewal | After the renewal | Before the renewal | After the renewal | Before the renewal | After the renewal | At the end |
| Control | 24.3 | 24.7 | 24.7 | 24.1 | 24.5 | 24.3 | 24.3 | 24.3 |
| 0.306 | 24.7 | 24.7 | 24.7 | 24.1 | 24.5 | 24.3 | 24.4 | 24.3 |

Unit: °C

Table 4 LC₅₀ to Medaka

| Exposure duration | LC ₅₀ (mg/L) | 95% confidence interval (mg/L) (Slope of the dose-response curve) | Statistical procedure used for determination of LC ₅₀ |
|-------------------|-------------------------|--|---|
| 24-hour | >0.306 | - (-) | - |
| 48-hour | >0.306 | - (-) | - |
| 72-hour | >0.306 | - (-) | - |
| 96-hour | >0.306 | - (-) | - |

- : Not obtained

Appendix 1

Chemical characteristics of dilution water

Chemical characteristics of dilution water (Sampling on January 9, 2007)

| Parameter | Unit | Results | Lower limit of determination |
|--|------|------------|------------------------------|
| Total hardness (as CaCO ₃) | mg/L | 41.9 | 0.1 |
| Suspended solid | mg/L | < 1 | 1 |
| pH | — | 7.9 (22°C) | — |
| Total organic carbon | mg/L | 0.2 | 0.1 |
| Chemical oxygen demand | mg/L | 0.7 | 0.5 |
| Residual chlorine | mg/L | < 0.02 | 0.02 |
| Ammonium ion | mg/L | 0.01 | 0.01 |
| Total cyan | mg/L | < 0.01 | 0.01 |
| Alkalinity | mg/L | 35 | 1 |
| Electric conductivity | mS/m | 18.3 | — |
| Organic phosphorous | mg/L | < 0.1 | 0.1 |
| Alkylmercury | mg/L | < 0.0005 | 0.0005 |
| Mercury | mg/L | < 0.0005 | 0.0005 |
| Cadmium | mg/L | < 0.001 | 0.001 |
| Chromium (VI) | mg/L | < 0.02 | 0.02 |
| Lead | mg/L | < 0.005 | 0.005 |
| Arsenic | mg/L | < 0.001 | 0.001 |
| Boron | mg/L | 0.08 | 0.02 |
| Fluorine | mg/L | < 0.1 | 0.1 |
| Iron | mg/L | < 0.01 | 0.01 |
| Copper | mg/L | < 0.005 | 0.005 |
| Cobalt | mg/L | < 0.001 | 0.001 |
| Manganese | mg/L | < 0.01 | 0.01 |
| Zinc | mg/L | < 0.01 | 0.01 |
| Aluminum | mg/L | 0.033 | 0.001 |
| Nickel | mg/L | < 0.001 | 0.001 |
| Silver | mg/L | < 0.0001 | 0.0001 |
| Sulfate ion | mg/L | 3.9 | 0.1 |
| Chloride ion | mg/L | 16 | 1 |
| Sodium | mg/L | 14.3 | 0.01 |
| Potassium | mg/L | 3.7 | 0.01 |
| Calcium | mg/L | 11.5 | 0.01 |
| Magnesium | mg/L | 3.2 | 0.01 |
| 1,2-dichloropropane | mg/L | < 0.0001 | 0.0001 |
| Chlorothalonil | mg/L | < 0.0001 | 0.0001 |
| Propyzamide | mg/L | < 0.0001 | 0.0001 |
| Chlornitrofen | mg/L | < 0.0001 | 0.0001 |
| Simazine | mg/L | < 0.001 | 0.001 |
| Thiobencarb | mg/L | < 0.0001 | 0.0001 |
| Diazinon | mg/L | < 0.0001 | 0.0001 |
| Isoxathion | mg/L | < 0.0001 | 0.0001 |
| Fenitrothion | mg/L | < 0.0001 | 0.0001 |
| EPN | mg/L | < 0.0001 | 0.0001 |
| Dichlorvos | mg/L | < 0.0001 | 0.0001 |
| Iprobenfos | mg/L | < 0.0001 | 0.0001 |
| PCB | mg/L | < 0.0005 | 0.0005 |

Appendix 2

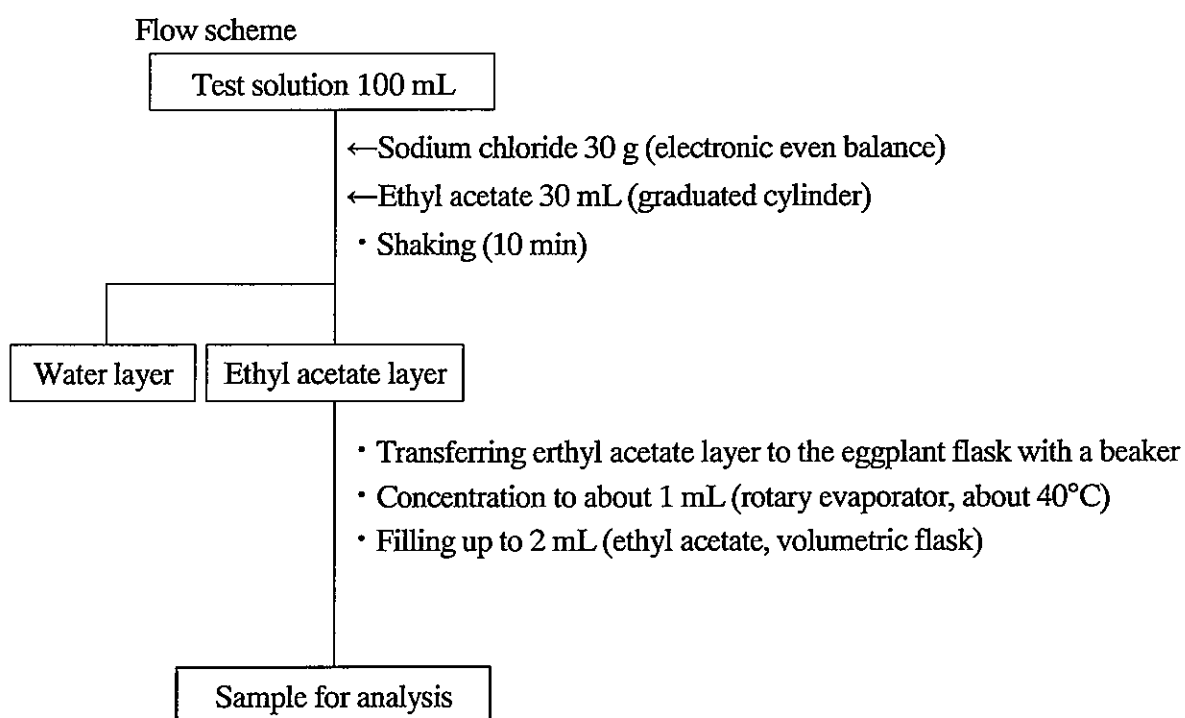
Analytical method and measured concentration of test item and ^{13}F -EtOH (hydrolyzed product)

1. Analysis of test solution

By the result of preliminary study, it was expected that the test item was hydrolyzed into 13F-EtOH during the exposure. Therefore, the concentration of the test item and 13F-EtOH were measured.

2. Pretreatment of test solution

The test solutions sampled were pretreated according to the following flow scheme to prepare the sample for analysis.



3. Method of analysis

The pretreated samples for analysis were quantitatively analyzed by gas chromatography (GC) under the following conditions to determine the concentrations of the test item and 13F-EtOH (hydrolyzed product). The concentrations of the test item and 13F-EtOH in each sample for GC analysis were determined on the basis of a comparison of the peak area on the chromatogram of the sample solution with that of a standard solution. The samples that the concentration of the test item and 13F-EtOH exceeded the range of the calibration curve were diluted to fall within the range and analyzed. Some chromatograms obtained are shown in Appendix 3.

Analytical conditions

| | |
|------------------|---|
| Instrument | Gas chromatograph Hewlett Packard HP 6890 Series GC System |
| Auto injector | Hewlett Packard HP6890 Series |
| Detector | Flame ionization detector (FID) |
| Column | DB-WAX film thickness 0.50 μm (Agilent Technologies) 30 m \times 0.32 mm I.D. Fused silica |
| Column temp. | 40°C (5 min) $\xrightarrow{\textcircled{1}}$ 150°C (0 min) $\xrightarrow{\textcircled{2}}$ 240°C (2 min) |
| Temp. rate | ①15°C/min ②50°C/min |
| Injection temp. | 200°C |
| Carrier gas | Helium |
| Column flow | 1.8 mL/min |
| Hydrogen | 40.0 mL/min |
| Air | 400 mL/min |
| Injection volume | 2 μL |
| Inlet mode | Splitless |
| Purge flow | 20.0 mL/min |
| Purge time | 0.50 min |
| Detector | |
| Temp. | 240°C |
| Sensitivity | Range 2 ⁰ |

4. Preparation of standard solution

The standard solutions to determine the concentrations of the test item and 13F-EtOH in the sample for analysis were prepared as follows. The standard solution was each prepared with correcting by the purity (99.7%) of the test item and the purity (99.8%) of 13F-EtOH.

The test sample of 100.3 mg was precisely weighed with an electronic balance and dissolved in ethyl acetate to obtain 1000 mg/L solution of the test item. The reference standard for 13F-EtOH component analysis (the test sample of study number 94232-94234) of 100.2 mg was precisely weighed with an electronic balance and dissolved in ethyl acetate to obtain 1000 mg/L solution of 13F-EtOH. The test item solution was diluted with ethyl acetate to prepare 25.0 mg/L (as the concentrations of the test item and 13F-EtOH) of test item and 13F-EtOH solution, after 13F-EtOH solution was added. The solution was diluted with ethyl acetate to prepare 2.50 mg/L (as each concentrations of the test item and 13F-EtOH) of standard solution.

5. Calibration curve

The standard solutions of 0.250, 1.25, 2.50 and 5.00 mg/L (as the concentrations of the test item and 13F-EtOH) were prepared by the same procedure as described in section 4. These solutions were analyzed according to the quantitative analytical conditions described in section 3. The calibration curves were drawn from the relationship between the concentrations of standard solution (the test item and 13F-EtOH) and the peak area on the chromatogram, and the determination was confirmed. The calibration curve is shown in Appendix 3. The determination limit of the test item was the lowest concentration of the standard solution (0.250 mg/L) within the range of the calibration confirmed. Therefore, the determination limit of the test item in the test solution was 0.00606 mg/L in consideration of pretreatment. The determination limit of 13F-EtOH was the lowest concentration of the standard solution (0.250 mg/L) within the range of the calibration confirmed. Therefore, the determination limit of 13F-EtOH in the test solution was 0.00604 mg/L in consideration of pretreatment.

6. Recovery test and blank test

6.1 Method

The recovery test was conducted by adding the test item solution (prepared with acetone) to dechlorinated tap water according to pretreatment of test solution described in section 2. Similarly, the recovery test was conducted by adding 13F-EtOH solution (prepared with acetone) to dechlorinated tap water. The blank test was also conducted using dechlorinated tap water (added acetone) without the test item and 13F-EtOH in the same way as the recovery test. The recovery test and the blank test were performed in duplicate.

| | |
|-------------------------------|--------------------|
| Amount of the test item added | 5.00 μg |
| Amount of 13F-EtOH added | 5.00 μg |

6.2 Result

As a result of analysis by the method of section 6.1, no peak of the test item and 13F-EtOH appeared in the chromatogram of the blank test. Two individual recovery rates and their average on the analytical procedure are shown below. The averages of recovery rate were used as correction factor, for the determination of the test item and 13F-EtOH concentrations in the test solutions.

Recovery rate of the test item for pretreatment
81.9%, 83.0% average 82.5%

Recovery rate of 13F-EtOH for pretreatment
82.6%, 83.0% average 82.8%

7. Results of measurement

The results of the measured concentrations of the test item and 13F-EtOH in the test solutions are shown below.

Appendix table 2-1 Measured concentrations of test item in test solutions

| Nominal concentration (mg/L) | Measured concentration of test item (mg/L) (Percentage of measured concentration versus that at each preparation %) | | | | | | | | Geometric mean |
|------------------------------|--|--------------------|-------------------|--------------------|-------------------|--------------------|-------------------|-----------------|----------------|
| | At the start | 24 hours | | 48 hours | | 72 hours | | At the end | |
| | | Before the renewal | After the renewal | Before the renewal | After the renewal | Before the renewal | After the renewal | | |
| Control | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. | |
| 100 | 0.409 | 0.190 (46.5) | 1.15 | 0.204 (17.8) | 0.391 | 0.0689 (17.6) | 0.855 | 0.183 (21.4) | 0.306 |

n.d. : <0.00606 mg/L

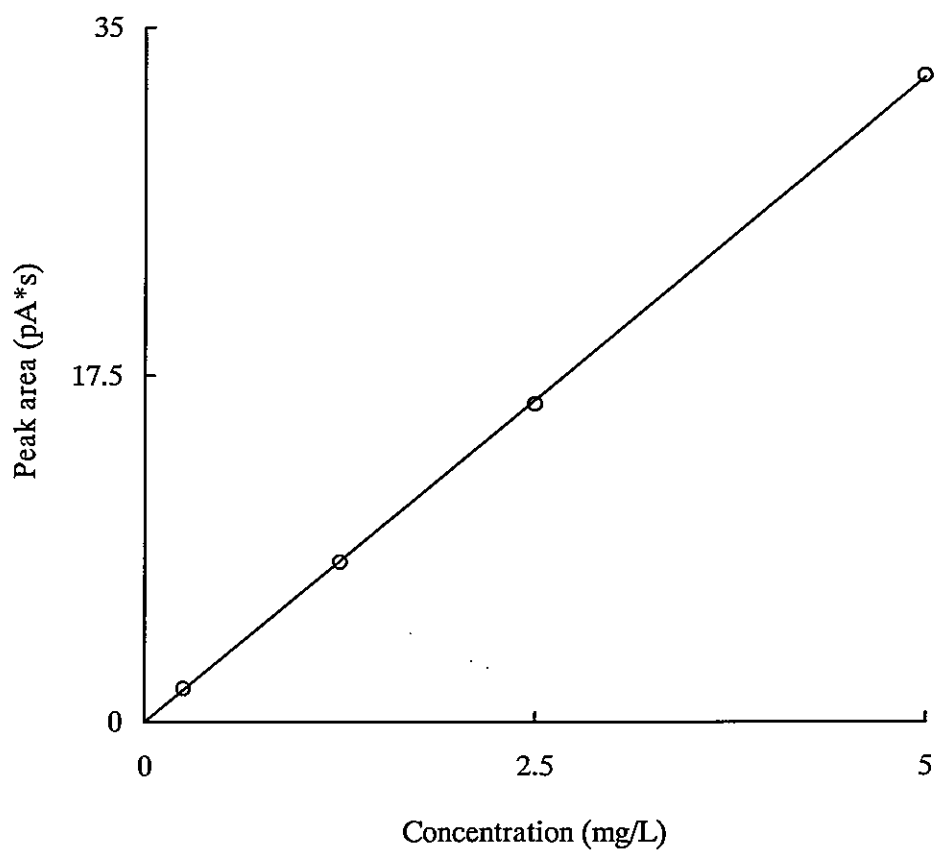
Appendix table 2-2 Measured concentrations of 13F-EtOH in test solutions

| Nominal concentration (mg/L) | Measured concentration 13F-EtOH (mg/L) | | | | | | | At the end |
|------------------------------|--|--------------------|-------------------|--------------------|-------------------|--------------------|-------------------|------------|
| | At the start | 24 hours | | 48 hours | | 72 hours | | |
| | | Before the renewal | After the renewal | Before the renewal | After the renewal | Before the renewal | After the renewal | |
| Control | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. |
| 100 | 0.0179 | 0.0811 | 0.0223 | 0.107 | 0.0388 | 0.181 | 0.0442 | 0.284 |

n.d. : <0.00604 mg/L

Appendix 3

Calibration curve and chromatogram



$$y = 6.49x$$

$$r = 1.00$$

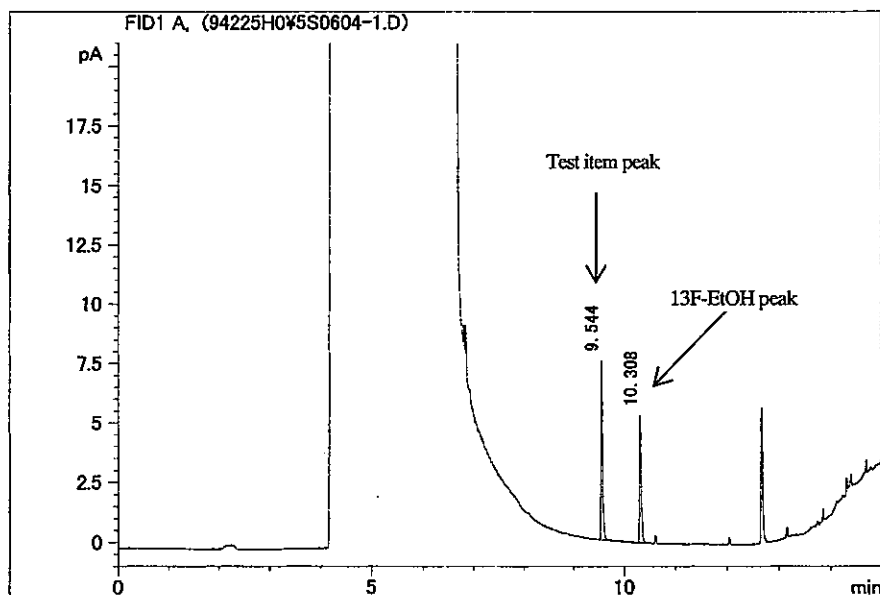
| Concentration (mg/L) | Peak area (pA*s) |
|----------------------|------------------|
| 0.250 | 1.667 |
| 1.25 | 8.062 |
| 2.50 | 16.015 |
| 5.00 | 32.546 |

Appendix figure 3-1 Calibration curve of 13F-SFA for analysis by GC.

Standard solution 2.50 mg/L

Study No. 94225

測定オペレータ : R. Adachi
 メソッド名 : C:\HPCHEM\1\METHODS\94223.M
 生データファイル名 : C:\HPCHEM\1\DATA\94225\H0\5S0604-1.D
 サンプル名 : std 2.50mg/L



| # | メインピーク面積 | メインピーク高さ | 実測 RT |
|---|----------|----------|--------|
| 1 | 16.290 | 7.518 | 9.544 |
| 2 | 12.093 | 5.330 | 10.308 |

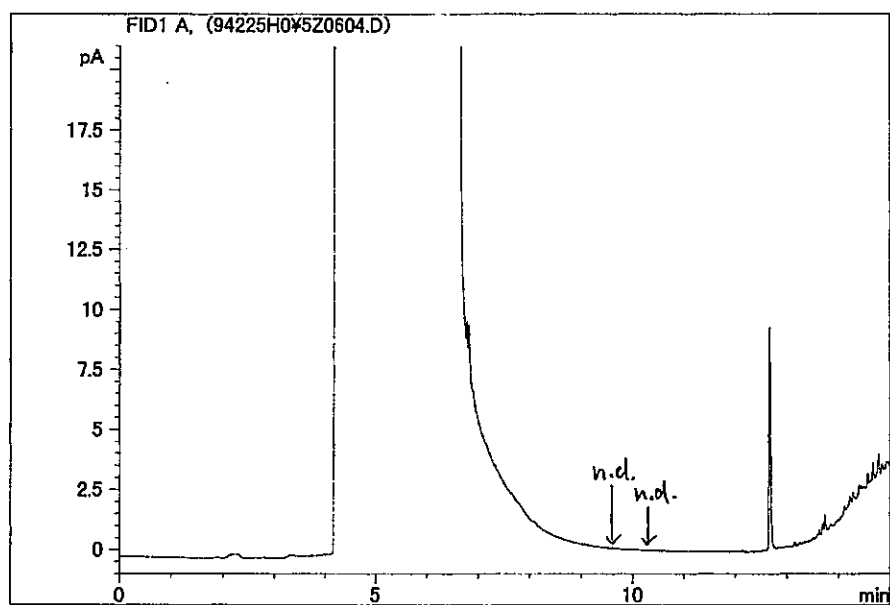


Appendix figure 3-2-1 GC chromatogram at start of exposure.

Control

Study No. 94225

測定オペレータ : R. Adachi
 メソッド名 : C:\HPCHEM\1\METHODS\94223.M
 生データファイル名 : C:\HPCHEM\1\DATA\94225H0\5Z0604.D
 サンプル名 : Cont. (*50)H0h



| # | ピーク面積 | ピーク高さ | 実測 RT |
|---|-------|-------|-------|
|---|-------|-------|-------|

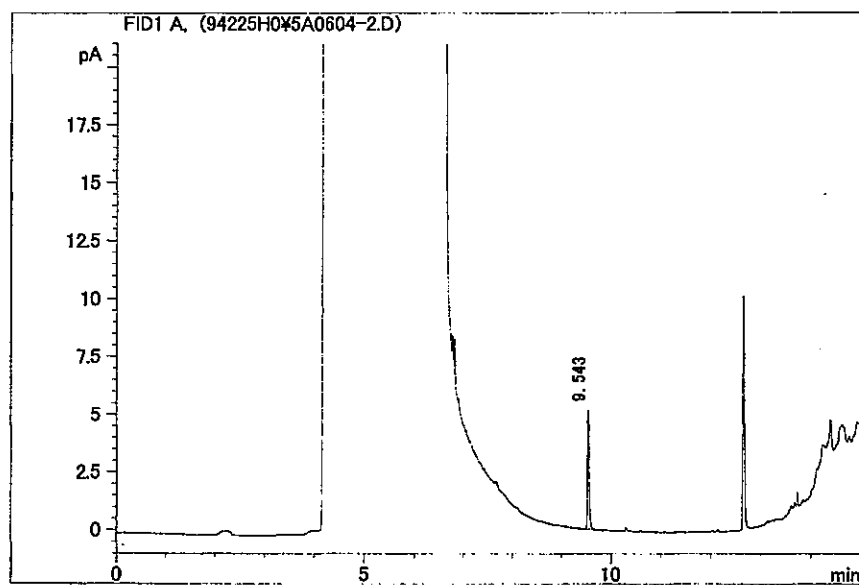


Appendix figure 3-2-2 GC chromatogram at start of exposure.

100 mg/L (Nominal concentration)

Study No. 94225

測定オペレータ : R. Adachi
 メソッド名 : C:\VHP\CHEM\1\METHODS\94223.M
 生データファイル名 : C:\VHP\CHEM\1\DATA\94225\H0\5A0604-2.D
 サンプル名 : 100 (*50D10) H0h



| # | メインピーク面積 | メインピーク高さ | 実測 RT |
|---|----------|----------|-------|
| 1 | 11.000 | 5.152 | 9.543 |

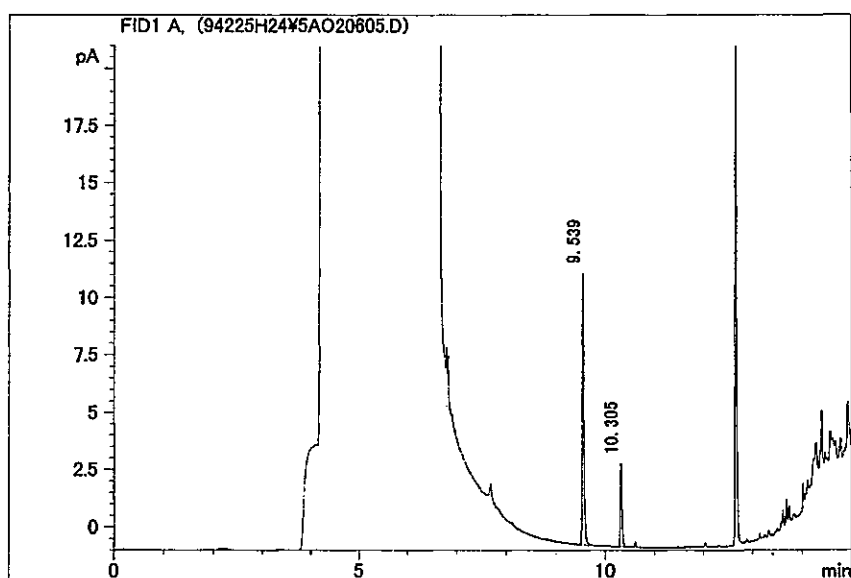


Appendix figure 3-2-3 GC chromatogram at start of exposure.

100 mg/L (Nominal concentration)

Study No. 94225

測定オペレータ : R. Adachi
メソッド名 : C:\VHPCHEM\1\METHODS\94223.M
生データファイル名 : C:\VHPCHEM\1\DATA\94225H24\5A020605.D
サンプル名 : 100 (*50D2)H24o



| # | メインピーク面積 | メインピーク高さ | 実測 RT |
|---|----------|----------|--------|
| 1 | 26.011 | 11.871 | 9.539 |
| 2 | 8.280 | 3.632 | 10.305 |

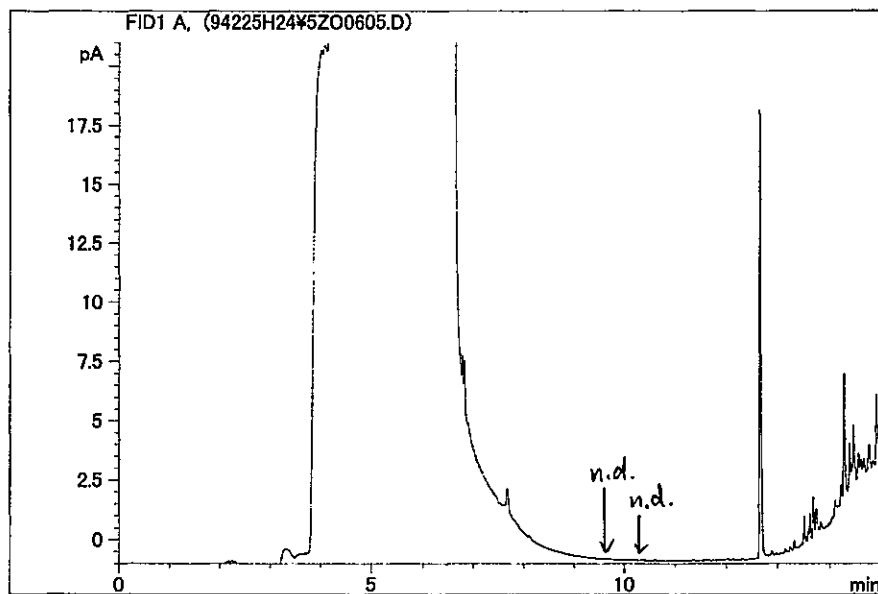


Appendix figure 3-3-3 GC chromatogram before renewal at 24 hours.

Control

Study No. 94225

測定オペレータ : R. Adachi
 メソッド名 : C:\HPCHEM\1\METHODS\94223.M
 生データファイル名 : C:\HPCHEM\1\DATA\94225H24\5Z00605.D
 サンプル名 : Cont. (*50)H24old



| # | ピーク面積 | ピーク高さ | 実測 RT |
|---|-------|-------|-------|
|---|-------|-------|-------|

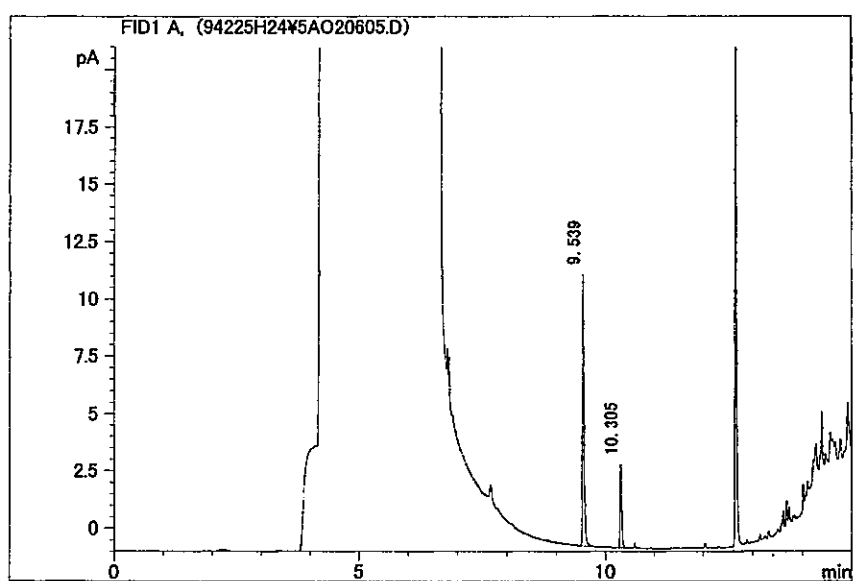


Appendix figure 3-3-2 GC chromatogram before renewal at 24 hours.

100 mg/L (Nominal concentration)

Study No. 94225

測定オペレータ : R. Adachi
 メソッド名 : C:\HPCHEM\1\METHODS\94223.M
 生データファイル名 : C:\HPCHEM\1\DATA\94225H24\5A020605.D
 サンプル名 : 100 (+50D2) H24o



| # | ピーク面積 | ピーク高さ | 実測 RT |
|---|--------|--------|--------|
| 1 | 26.011 | 11.871 | 9.539 |
| 2 | 8.280 | 3.632 | 10.305 |



Appendix figure 3-3-3 GC chromatogram before renewal at 24 hours.

Appendix 4

Solubility in dilution water

1. Title

Solubility of test item in dilution water

2. Objective

The objective of this study is to estimate the solubility of the test item to dilution water.

3. Outline

Test item mixed with dilution water was stirred for 24 hours under the test temperature. After clearing insoluble matter, solubility was analyzed.

4. Performance of test

4.1 Test equipments and instruments

Water bath : Plastic tank
Warming/cooling unit (Type HCA250, Sato craft)
Mixing apparatus : Magnetic stirrer
Vessel : Devised glass container
(Interior volume : About 600 mL)

4.2 Test conditions

- (1) Test temperature : $24 \pm 1^\circ\text{C}$
- (2) The number of measurement : Once (after the mixture was stirred for 24 hours)
- (3) Dilution water : Dechlorinated tap water
- (4) Repetition : $n=3$ (Sample-1, Sample-2 and Sample-3)

4.3 Test procedures

(1) Test sample and dilution water were mixed in a devised glass container to prepare about 100 mg/L* solution and sealed without headspace.

* The additive amount (38.6 μL) was calculated from the density of the test item (1.554 g/cm^3).

(2) The test solution was stirred slowly with a magnetic stirrer under the test temperature in a water bath.

(3) After the solution was stirred for 24 hours, the flask was settled in a water bath for about 1 hour.

(4) After settling, the samples were quantitatively analyzed to measure the concentration of the test item and 13F-EtOH (hydrolyzed product).

4.4 Analysis of test solution

(1) Pretreatment of test solution

The middle layer of the test solutions were collected carefully from sampling spout of the devised glass container by syringe. The collected solution was pretreated according to the flow scheme described in Appendix 2 2. Pretreatment of test solution.

(2) Method for analysis

See Appendix 2 3. Method of analysis.

4.5 Preparation of standard solution

See Appendix 2 4. Preparation of standard solution.

4.6 Calibration curve

See Appendix 2 5. Calibration curve.

4.7 Recovery test and blank test

See Appendix 2 6. Recovery test and blank test

5. Results

Measured solubility of the test item in dilution water was 0.126 mg/L. In addition, the measured concentration of 13F-EtOH was under determination limit (0.0630 mg/L). The results of analyses are shown below.

Appendix table 4-1 Value measured after stirring for 24 hours (test item)

| Sample name | Measured value (mg/L) | Arithmetic mean (mg/L) |
|-------------|-----------------------|------------------------|
| Sample-1 | 0.140 | 0.126 |
| Sample-2 | 0.122 | |
| Sample-3 | 0.115 | |

Appendix table 4-2 Value measured after stirring for 24 hours (13F-EtOH)

| Sample name | Measured value (mg/L) | Arithmetic mean (mg/L) |
|-------------|-----------------------|------------------------|
| Sample-1 | 0.0678 | 0.0630 |
| Sample-2 | 0.0642 | |
| Sample-3 | 0.0570 | |

Additional data

Results of preliminary studies

1. Solubility of test item in dilution water

It was expected that the solubility of the test item in dilution water was below 100 mg/L, therefore, the measurement of the solubility of the test item in dilution water was conducted. The following preliminary study 2 was performed in algae growth inhibition test (Study number: 94223).

1) Preliminary study 1 for measurement of solubility

(1) Method

Since the test item was expected to be volatile due to the chemical structure, the test item and the dilution water (dechlorinated tap water) were mixed and gently stirred in a devised glass container under closed system with no head space and test temperature ($24\pm 1^\circ\text{C}$) for 24 and 48 hours. And then the middle layer was sampled after settling for about 1 hour. Centrifugation and filtration with a membrane filter for removal of insoluble substance were not demonstrated, because these methods caused the decrease of test item concentration. The concentration of the test item in the collected sample was analyzed after the pretreatment by gas chromatography (GC) (n=2).

(2) Result

| Nominal concentration (mg/L) | Measured concentration (mg/L) | |
|---------------------------------|-------------------------------|------------------|
| | 24-hour stirring | 48-hour stirring |
| Approx. 100 (Sample-1) | 0.268 | - |
| Approx. 100 (Sample-2) | 0.153 | - |
| Approx. 100 (Sample-3) | - | 0.257 |
| Approx. 100 (Sample-4) | - | 0.172 |

Solubility of test item in dilution water was around 0.1 to 0.3 mg/L.

2) Preliminary study 2 for measurement of solubility

(1) Method

Since the test item was forecasted to be hydrolyzed into 13F-EtOH, the solubility of the test item in dilution water and 13F-EtOH (the test item of study number 94232-94234) were measured at the same time. Firstly, the test item and the dilution water (dechlorinated tap water) were mixed and gently stirred in a devised glass container under closed system with no head space and test temperature ($24\pm 1^\circ\text{C}$) as condition of fish acute toxicity test for 24 and 48 hours. And then the middle layer was sampled after settling for about 1 hour. The concentration of the test item and 13F-EtOH in the collected sample were analyzed after the pretreatment by gas chromatography (GC) (n=2).

(2) Result

Measured concentration of the test item

| Nominal concentration (mg/L) | Measured concentration of test item (mg/L) | |
|---------------------------------|--|-----------------------|
| | Stirring for 24 hours | Stirring for 48 hours |
| Approx. 100 (Sample-1) | 0.392 | - |
| Approx. 100 (Sample-2) | 0.609 | - |
| Approx. 100 (Sample-3) | - | 0.0923 |
| Approx. 100 (Sample-4) | - | 0.0856 |

Measured concentration of 13F-EtOH

| Nominal concentration (mg/L) | Measured concentration of 13F-EtOH (mg/L) | |
|---------------------------------|---|-----------------------|
| | Stirring for 24 hours | Stirring for 48 hours |
| Approx. 100 (Sample-1) | 0.0843 | - |
| Approx. 100 (Sample-2) | 0.0721 | - |
| Approx. 100 (Sample-3) | - | 0.552 |
| Approx. 100 (Sample-4) | - | 0.291 |

The solubility of the test item in dilution water at 24-hour stirring was higher than the value of preliminary study 1 for measurement of solubility, because devised glass container was not used but Erlenmeyer flask, so the test solution was contaminated with the suspended test sample at the sampling. The measured concentration of 13F-EtOH (hydrolyzed product) was generated 0.07 to 0.1 mg/L at 24-hour stirring, and 0.2 to 0.6 mg/L 48-hour stirring.

3) Summary of preliminary study for measurement of solubility

Since the test item was expected to volatile due to the chemical structure, the test solution was stirred gently in a devised glass container under closed system with no head space. By the results of preliminary study 1, the solubility of the test item in dilution water was around 0.1 to 0.3 mg/L. And then, the solubility of the test item in dilution water was within twice to the difference of the analytical results at 24 and 48-hour stirring. It considered that the solubility of the test item in dilution water was the solubilities of the test item at 24 and 48-hour stirring were about the same value. On the other hand, by the result of preliminary study 2, the measured concentration of 13F-EtOH (hydrolyzed product) at 24-hour stirring was around 0.08 mg/L, but it was greatly increased to 0.2 to 0.6 mg/L at 48-hour stirring.

From the results mentioned above, in definitive study the devised glass container would be used for the preparation in definitive study. It was decided that the test solution was stirred for 24 hours, because the solubilities of the test item at 24 and 48-hour stirring were about the same value, and the production amount of hydrolyzed product was low.

2. Effect on test organism

1) Preliminary study

(1) Method

After the test sample was added into the dilution water filled in Erlenmeyer flask with micro volumeter (Eppendorf Co., Ltd) to produce about 100 mg/L as nominal concentration, the flask was immediately sealed with a plug to produce without head space. The solution was gently stirred by magnetic stirrer for about 48 hours to prepare the dispersed solution with suspended test item. This preliminary study was conducted by closed system without head space, since the test item was suspected to volatile. The saturation concentration of the test item in the test solution was also carried out.

(2) Result

| Nominal concentration (mg/L) | Left column : Cumulative mortality (%) Right column : Existence of abnormal response (abnormalities : *, no abnormalities : -) | | | | | | | | | |
|------------------------------|---|---|----------|---|----------|---|----------|---|----------|---|
| | 3 hours | | 24 hours | | 48 hours | | 72 hours | | 96 hours | |
| Control | 0 | - | 0 | - | 0 | - | 0 | - | 0 | - |
| 101 | 0 | - | 0 | - | 0 | - | 0 | - | 0 | - |

Type of test : Semi-static (renewal at every 24 hours)

Number of organisms/volume of test solution: Five fish/about 3.2 L

Aeration: Not conducted

<Measured concentration of test item in test solution>

| Nominal concentration (mg/L) | Measured concentration (mg/L) (percentage of measured concentration at start) | |
|------------------------------|--|-----------------|
| | At the start | After 24 hours |
| Control | n.d. | n.d. |
| 101 | 0.513 | 0.194 (37.9) |

n.d. : <0.00572 mg/L

The measured concentration of the test item in the test solution remarkably decreased at 24 hours after the start.

2) Summary of effect on test organisms (preliminary study)

No effect of the test item on the test organisms was observed in the preliminary study using the dispersed test solution prepared by mixing the test item and the dilution water to produce a limit concentration (100 mg/L) in "Testing Methods for New Chemical Substances", and then the NOEC (No Observed Effect Concentration) was expected to be greater than the limit concentration prescribed in the test method.

3. Result of preliminary study (Summary)

In the preliminary study for measurement of solubility, the concentration of the test item in the dilution water was supposed to reach the saturated value by stirring for 24 hours. The hydrolyzed product (13F-EtOH) of the test item gradually increased with time.

In the preliminary study for effect on test organism, no adverse effect was observed in the saturated solution prepared with 48-hour stirring of general procedure. However, while both the concentrations prepared by 24-hour and 48-hour stirring were almost the same value as the solubility obtained in the preliminary study for measurement of solubility, the hydrolyze product was produced more in the saturated solution at 48- hour stirring than that at 24-hour. Therefore, I thought that it was proper to use the test solution containing less concentration of the hydrolyzed product in the case of the similar concentration in the saturated solution of the test item, and then the definitive study planed to conduct using the saturated test solution prepared by 24-hour stirring. It was expected that no effect of the test item on the organisms would be observed in the saturated test solution prepared by 24-hour stirring.

4. Operation of definitive study

1) Preliminary study

Based on the result of the preliminary study, the measurement of the solubility was carried out using the solution prepared by mixing the test sample and the dilution water to produce approximately 100 mg/L, and by stirring gently for 24 hours under closed system and $24\pm 1^{\circ}\text{C}$. For removal of insoluble substance, the procedure of centrifugation and filtration was not employed. Instead of using their procedure, to minimize insoluble substance it was removed by taking out from the middle layer of the solution settled for 1 hour after cease of stirring. The concentration of the test item and 13F-EtOH, which was the hydrolyzed product of the test item, was measured for the prepared test solution.

2) Definitive study

The definitive study was planned to be carried out using the nominal test concentration of an upper limit (100 mg/L) of exposure level in the test method, and using the middle layer of the dispersed test solution prepared by stirring for about 24 hours, and a control. The study was conducted under the condition of the replacement of the test solution with once a day and closed system. The test solution was prepared as follows; After the test sample was added, in terms of volume using the density, into the dilution water filled in Erlenmeyer flask with micro volumeter (Eppendorf Co., Ltd) to prepare test solution of 100 mg/L as nominal concentration, the flask was immediately sealed with a plug not to produce head space. The solution was stirred by magnetic stirrer for about 24 hours to produce dispersed solution with suspended test item. After cease of stirring, the solution was settled for 1 hour under about $24\pm 1^{\circ}\text{C}$ and then test solution was prepared by taking out from the middle layer of the settled solution. The measurement of the test item and 13F-EtOH in the test solution was carried out at the start of the exposure, before and after the renewal, and at the end of the exposure.